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Cytological characterization of *Jatropha curcas* callus in different periods of cultivation

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Abstract – The aimed was characterization and determining the time for *Jatropha curcas* callus subculture. The pattern of *J. curcas* callus development is sigmoidal with 5 distinct phases, and transplantation should be performed at the end of the deceleration phase (approximately day 28). During callus development, it was observed that at the onset of growth in the exponential phase the cells were internally disorganized; in the linear phase, respiratory metabolism was resumed through reorganization of the first mitochondria; and by the mid-deceleration phase, the cells were entirely active, and several organelles were detected. This organizational status was maintained throughout the stationary phase during which somatic pre-embryos were identified. At the end of the stationary phase, intracellular disruption began, and the cells entered senescence, which characterized the decline phase for the *J. curcas* calli growth curve.

Key words: In vitro, kinetics and ultrastructure.

INTRODUCTION

The demand for alternative sources of energy is global, and alternative fuels, such as bioethanol and biodiesel, may relieve the problems caused by fossil fuel consumption. Among the available oil-producing plants, *J. curcas* has been gaining agronomic importance, which is demonstrated by recent cytogenetic characterization (Carvalho et al. 2008, Dahmer et al. 2009) and genome sequencing (Sato et al. 2010) that facilitate more applied research with this species.

Tissue culture represents an area of biotechnology research that has significantly progressed for *J. curcas*. The first reports of the cultivation of *J. curcas* in vitro get along with Sujatha and Mukta (1996), which tested various explants, including leaves, petioles and hypocotyls in 0.5 mg L⁻¹ BAP (benzylaminopurine) and 1.0 mg L⁻¹ IBA (indolebutyric acid). A recently published patent (US 7,932,086 B2) disclosed micropropagation of *J. curcas* on a commercial scale (Murali et al. 2011). Further, according to Leela et al. (2011) and Sharma and Chauhan (2011), micropropagation of *J. curcas* produces genetically stable plants when it is limited to seven subcultures. Therefore, tissue culture for *J. curcas* micropropagation is in the process of consolidation (Sardana et al. 2000, Jha et al. 2007, Nunes et al. 2008).

An additional sub-area for further research is *in vitro* regeneration because it is the technique that regenerates organs and tissues in transformed plants; this technique has been used in *J. curcas* studies with both biolistic and *Agrobacterium* transformation (Joshi et al. 2011, Misra et al. 2011).

In vitro regeneration has several restrictions including subculture timing in pathways mediated by callus. This information is often not fully elucidated in manuscripts on *in vitro* regeneration of *J. curcas* (Qin et al. 2004, Kaewpoo and Te-Chatob 2009, Kumar and Reddy 2010, Kumar et al. 2011); therefore, the average subculture time should be determined for the morphogenetic processes in this species.

The callus growth curve is a functional tool that provides the average time for subculturing, which is useful because in the upward growth phase of the curve, cells have a high capacity for division, and after this period, they enter the stationary phase. In the stationary phase, a high number of cells have a lower capacity for division, and these cells enter the cell differentiation stage (Pereira et al. 2007). Therefore, using the callus growth curve, the crop transplantation time can be identified in addition to the best phases to determine development kinetics and maximize cell replication (Abade et al. 2010).

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The callus growth curve can also assist in monitoring development when it is linked to an additional tool, such as electron microscopy. According to Fernando et al. (2007), electron microscopy is a valuable tool used to elucidate the *in vitro* regeneration process.

There are only brief reports of electron microscopy use for *J. curcas*, with only two records being available in the literature. The first describes the morphological differences between browning and non-browning *J. curcas* calli cultured *in vitro* (He et al. 2009), and the latter characterizes flower development in *J. curcas* (Wu et al. 2011). There are no reports in which electron microscopy was used to discern the morphogenetic processes for *J. curcas*, and according to Varshney et al. (2011), this approach would significantly contribute to elucidating an *in vitro* culture system.

Consequently, the study herein uses techniques and tools to determine and reduce the callus mass incubation periods as well as to investigate their development and improve morphogenetic success. Thus, the study herein was aimed at determining the time for *J. curcas* callus subculture using the callus growth curve and monitoring their development by evaluating cellular and intracellular characteristics using ultrastructural analyses.

MATERIAL AND METHODS

Plant material

The experiment described herein was conducted in the Plant Tissue Culture Laboratory of the Department of Agriculture at the Federal University of Lavras (Laboratório de Cultura de Tecidos Vegetais do Departamento de Agricultura da Universidade Federal de Lavras, UFLA).

The *J. curcas* seeds were generated *in vitro* using the method developed by Nunes et al. (2008) to produce a seedling with fully expanded cotyledons. The cotyledons were cut into 1 cm² segments using a scalpel in a sterile laminar flow hood, inoculated in an MS medium (Murashig and Skoog 1962), prepared by adding 30 g L⁻¹ of saccharose supplemented with 1 mg L⁻¹ of 2,4-D (2,4-dichlorophenoxyacetic acid), solidified with 5.5 g L⁻¹ of agar, and then autoclaved at 121 °C for 20 min at 1 atm. The cultures were stored in a metal cabinet to ensure complete darkness in a growth chamber at 25±2 °C.

The experiment followed a randomized design and was statistically analyzed using the Regression test with the Sisvar software (Ferreira 2011).

Callus growth curve

The *J. curcas* calli were subcultured twice at 30-day

intervals to ensure that the final explant for inoculation was a genuine callus with no leaf tissue. The callus growth curve was established after the second subculture by inoculating the aggregates in a medium with 0.29 g of fresh matter. They were then weighed for 112 days at seven-day intervals beginning at inoculation.

The experimental design was completely randomized with 16 treatments, consisting of the evaluation intervals (0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, 98, 105 and 112 days). Therefore, growth at 16 time periods was determined and characterized at the assessment intervals using three replicates; each replicate was an average of three tubes, and each tube contained one explant. The growth curve was plotted using the average of three replicates for each mass assessment. Concomitantly, the callus masses were evaluated ultrastructurally using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) for each growth period.

Ultrastructural analysis

To prepare and observe the samples for TEM and SEM, approximately 1 cm² sections from a callus mass were cut from each treatment and immersed in Karnovsky's fixative (modified Karnovsky's fixative, 2.5% glutaraldehyde, 2.5% formaldehyde in 0.05 M sodium cacodylate buffer, pH 7.2 and 0.001 M CaCl₂) for 24 h. Subsequently, the samples were pre-fixed in aldehyde and washed three times in 0.05 M cacodylate buffer for approximately 10 min and then immersed in 1% osmium tetroxide (OsO₄) in 0.05 M cacodylate buffer pH 7.2 for 1 h at room temperature in a fume hood. The samples fixed in OsO₄ were washed with distilled water and added to solutions with increasing acetone concentrations (25, 50, 75, 90 and 100%) for dehydration. They remained in each solution for approximately 10 min; however, they were immersed in the 100% solution three times.

For the TEM analysis, after dehydration in acetone, the samples were embedded in 30% and 70% resin for 8 h and 12 h, respectively. Subsequently, the samples were twice added to a 100% resin for 24 h each; the material was placed in a suitable mold, and it polymerized in an oven at 70 °C for 48 h. After ultramicrotomy, the ultra-thin sections were transferred to screens and contrasted using uranyl acetate and lead citrate. A Zeiss EM 109 Transmission Electron Microscope was used for observations and electron microscopy analysis.

For the SEM analysis, the material was dried in the critical point apparatus, the stubs were mounted, and the material was sputter-coated with gold. The prepared leaf material was observed, and electron micrograph images were taken using the Leo Evo 40 SEM.

RESULTS AND DISCUSSION

The kinetic pattern for a callus growth curve is sigmoidal and comprises lag, exponential, linear, deceleration, and stationary phases. This behavior was observed in several woody species, such as *Bertholletia excelsa* (Serra et al. 2000) and *Byrsonima intermedia* A. Juss. (Abbade et al. 2010). However, not all phases are necessarily observed; for example, Stein et al. (2010) did not identify the stationary phase in the *Inga vera* Willd callus.

For *J. curcas*, the lag phase was absent, and the decline phase was identified. Thus, *J. curcas* has five callus development phases: exponential, linear, deceleration, stationary, and decline (Figure 1).

A lag phase was not identified in the callus growth curve for *J. curcas*. As this is the preparation phase for cell division, it likely spanned less than 7 days and, consequently, was not identified because the assessment intervals were 7-days long. A secondary explant (callus) was used for kinetic evaluation because it had begun dividing. For example, Serra et al. (2000) demonstrated that the lag phase is extended when a leaf is used as the initial explant using *B. excelsa* leaves to initiate a growth curve and detecting a 30-day lag phase.

The callus growth curve for *J. curcas* began with an exponential phase in which cell division was already pronounced, and it remained in this phase from days 0 to 7. In contrast, the callus growth curve begins with the lag phase for *B. intermedia* (Abbade et al. 2010).

The linear growth period in which the calli decrease the rate of cell division and increase cellular area (Smith 1992) was observed between days 7 and 14. This brief period is due to the homogenous tissue, as it is a secondary explant

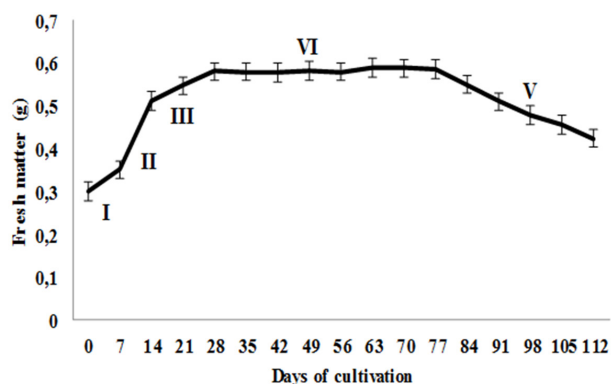


Figure 1. The callus growth curve from data obtained using *Jatropha curcas* leaf explants inoculated in MS medium supplemented with 1.0 mg L⁻¹ of 2,4-D. I. exponential growth phase (0-7 days); II. linear phase (7-14 days); III. deceleration phase (14-28 days); IV. stationary phase (28-77 days); and V. decline phase (after 77 days).

(Serra et al. 2000).

The deceleration phase composes the period from days 14 to 28. According to Smith (1992), calli must be subcultured during the deceleration phase because the cells do not divide by the mid-stationary phase. Therefore, *J. curcas* calli should be subcultured approximately 28 days after growth.

The stationary phase continued until day 77. It is worth noting that in this phase, growth ceased, but the development processes, such as differentiation, were active. At day 84, the *J. curcas* calli entered the decline phase, wherein development also ceased because the callus was in senescence.

Using TEM during the *J. curcas* callus growth curve, large vacuoles and a thin cell wall were observed from the day of inoculation to 7 days later (exponential phase). In general, the cells had few organelles, a disorganized cellular system, and a narrow cytoplasm, which the vacuole compressed against the cell wall (Figure 2a-b). According to Lodish et al. (2000), a low number of organelles was also reported for *B. intermedia* with embryogenic calli, which is characteristic of meristematic or embryonic cells during growth. Canhoto et al. (1996) also observed cytoplasm compression by the vacuole in cells with pro-embryogenic

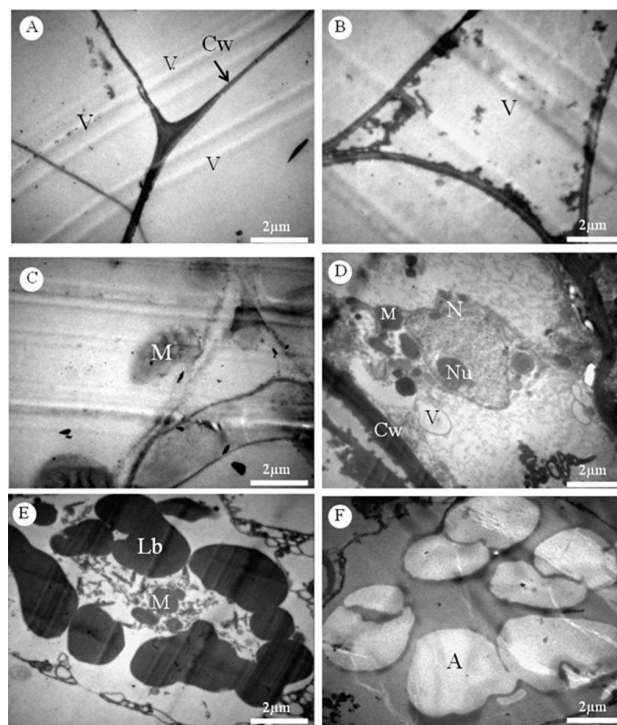


Figure 2. Electron micrographs of *Jatropha curcas* calli developed using 1 mg L⁻¹ of 2,4-D. a-b: exponential growth phase (0-7 days), large vacuoles (v) and a thin cell wall (Cw). c-d: deceleration phase (14-28 days), mitochondria (M) likely with cristae and fully organized cell with a nucleus (N), nucleolus (Nu), mitochondria (M), vacuole (V) and cell wall (Cw). e-f: stationary phase (28-77 days), lipid bodies (LB) and amyloplasts (A).

characteristics.

After 7 days of growth, cellular evolution was observed. The nucleus took shape, and at the end of the linear phase, the mitochondria formed, which demonstrated that respiratory metabolism in the callus mass was activated. Functional respiratory metabolism is important because the saccharose previously added to the culture medium is incorporated in glycolysis through respiration. Saccharose catabolism into smaller sugars produces the ATP needed to meet the cell's energy demand for its anabolic processes. With active respiratory metabolism in the callus, cell division is resumed, which renews the cellular complex in the callus mass. Thus, the linear growth phase in the *J. curcas* callus growth curve was observed during this period (7 days).

During the mid-deceleration phase (21 days), fully developed mitochondria were detected, and the mitochondrial cristae were visualized, which is key for aerobic respiration because it is where certain electron transport and ATP synthesis reactions occur (Figure 2c). The mitochondrial matrix, which contains several enzymes and other compounds involved in the Krebs cycle, is in the space enclosed by the inner membrane. The Krebs cycle is also essential for aerobic respiration because it generates NADH and FADH₂, which are precursors used in the electron transport chain (Semenza 2007).

Over time, the organizational status of entire cell improved. At the end of the deceleration phase (28 days), the nucleus contained a prominent nucleolus, mitochondria, small vacuoles, and a thick cell wall (Figure 2d). A thick wall indicates a decreased rate of cell division. According to Evert (2006), thick walls may hinder (retard and even prevent) cell division, which explains the stationary growth period observed for the *J. curcas* callus growth curve at the end of the deceleration phase. In this phase, lipid bodies were also observed (Figure 2e), which may function as reserves and can be converted into glucose as a substrate for respiration, which is commonly observed in seeds (Evert 2006).

Notably, the complete organizational status of the cell at 28 days validates the subculture period suggested by the *J. curcas* callus curve, and this approach can only be used when the organizational status of the cell is complete.

The intracellular characteristics generated during the deceleration phase were maintained throughout the stationary phase, and only at the end of this phase (after 77 days) was cellular disarray observed with many amyloplasts (Figure 2f). Häslér et al. (2003) also observed many amyloplasts in cells cultured *in vitro* for long periods and linked this observation to loss of cell regenerative capacity. Thus, long growth periods are disadvantageous for the *J. curcas* species because the crop loses vitality. A long growth period was

only used herein because it was required for the experiment.

The SEM photomicrographs show dense aggregates around the linear growth phase with primarily spherical cells. According to Na et al. (2007), both of these characteristics are typical for embryogenic cells; non-embryogenic complexes show the opposite characteristics (Figure 3a-b). The *J. curcas* calli form such aggregates because the inoculated callus mass is from two subcultures in MS medium that contains 1 mg.L⁻¹ de 2,4-D, which is a regulator that induces somatic embryogenesis (Zuo et al. 2002, George et al. 2008).

Through the end of the stationary phase, the cellular characteristics observed using this tool were the same as described previously, and at day 77, evidence of embryogenesis was observed, as the development process included two of the first phases of somatic embryo differentiation, the globular and cordiform embryo stages (Figure 3c-f). These structures did not continue to develop because the cells entered the decline phase, and as shown by TEM, they lost organization at the intracellular level due to senescence. The other stages of somatic embryo differentiation could be observed by following the methodological precepts for somatic embryogenesis, such as transferring the pre-embryos

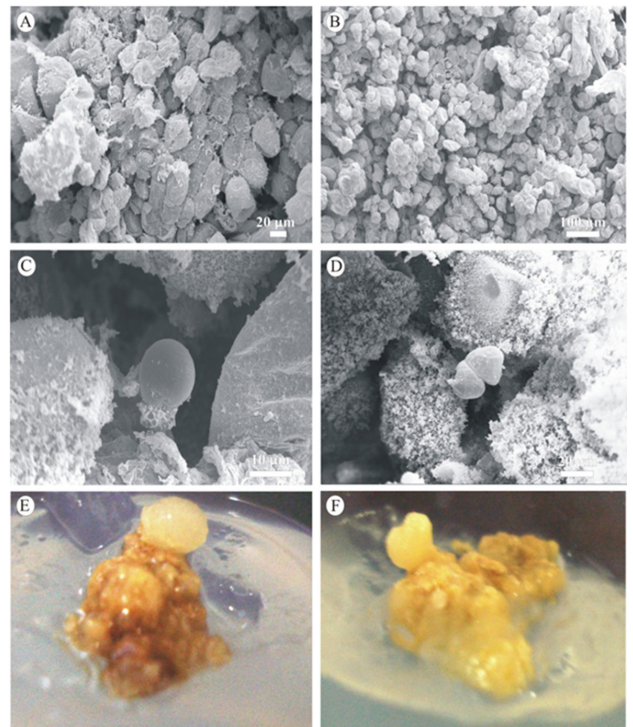


Figure 3. *Jatropha curcas* callus developed with 1 mg.L⁻¹ of 2,4-D. a-b: exponential growth phase (0-7 days), electron micrograph of the cells are isodiametric on the day of inoculation. c-e: stationary phase (28-77 days), details of the somatic embryos during the globular and cordiform stages.

to a suitable medium; however, this was not the main focus for the research herein.

Thus, using the analytical tools discussed herein, the callus growth curve was measured, and it was established that *J. curcas* callus masses should be subcultured at approximately day 28 of growth. TEM was used to validate this conclusion, as it showed that the cell was not completely organized during the earlier stages, and at later stages, the cell reached a stable phase and then senesced. By subculturing at approximately day 28, the morphogenetic expression potential of *J. curcas* callus can be explored.

J. curcas has five development phases and should be subcultured at the end of the deceleration phase (approximately day 28). At the onset of growth, cells from the *J. curcas* callus mass were disorganized at the intracellular level.

Caracterização citológica de calos de *Jatropha curcas* L. em diferentes períodos de cultivo

Resumo – Determinou-se o tempo de subcultivo de calos de *Jatropha curcas*. Uma curva de crescimento dos calos foi estruturada por 16 períodos de cultivo e para cada período foi feita análise ultraestrutural em microscopia eletrônica de transmissão e varredura. O padrão de desenvolvimento dos calos de *J. curcas* é uma sigmóide com cinco fases e é ao final da desaceleração que deve ser realizada a repicagem, em média aos 28 dias de cultivo. No início do cultivo dos calos, na fase exponencial, as células estão desorganizadas subcelularmente; na fase linear é retomado o metabolismo respiratório por reestruturação das primeiras mitocôndrias, e em meados da fase de desaceleração as células estão completamente ativas, podendo se detectar várias organelas. Este nível organizacional se mantém por toda a fase estacionária, durante a qual pré-embriões somáticos foram identificados. Ao final da fase estacionária começa a desestruturação subcelular; as células entram em processo de senescência, caracterizando a fase de declínio da curva de crescimento de calo de *J. curcas*.

Palavras-chave: In vitro, cinética e ultraestrutura.

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- Beginning with the linear phase, respiratory metabolism resumed function by restructuring the first mitochondria; at the end of the deceleration phase, all of the organelles were observed to be present, and full cellular activity was resumed. On average, this level of cellular organization is maintained for over 56 days, during which somatic pre-embryos were identified. After 84 days, the *J. curcas* callus cells senesced.

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